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Cis-9-octadecenoic acid from the rhizospheric bacterium *Stenotrophomonas maltophilia* BJ01 shows quorum quenching and anti-biofilm activities

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Quorum quenching (QQ) is an effective approach for the prevention of bacterial infections involving biofilms. This study reports the QQ and anti-biofilm activities of a rhizospheric bacterium identified as *Stenotrophomonas maltophilia* BJ01. The QQ activity was demonstrated using *Chromobacterium violaceum* CV026 as a biosensor. A maximum of 95% reduction in violacein production, a quorum sensing-regulated behavior, was observed. Gas chromatography–mass spectroscopy of the extract showed that the active compound was *cis*-9-octadecenoic acid, which was confirmed by electron-spray ionization–mass spectroscopy data. The extract also inhibited biofilm formation of *Pseudomonas aeruginosa* ATCC 9027 without affecting its growth. Scanning electron and atomic force microscopy showed architectural disruption of the biofilm when treated with the extract. This is the first report of the QQ and anti-biofilm activities of *cis*-9-octadecenoic acid isolated from any bacterium. It may have the potential to combat detrimental infections with *P. aeruginosa*. Further validation is required for any possible medical application.

Keywords: *Stenotrophomonas maltophilia*; *Chromobacterium violaceum* CV026; quorum quenching; *cis*-9-octadecenoic acid; anti-biofilm

Introduction

Bacteria exist in two different life forms, *viz*. as a biofilm and planktonic. A biofilm is the most successful form of life in the environment (Flemming & Wingender 2010). It represents a surface-associated microbial community embedded in extracellular polymeric substances (EPS) (O'Toole et al. 2000; Donlan & Costerton 2002; Shirtliff et al. 2002). Biofilms are involved in about 65% of all bacterial infections in humans according to the estimate of the Centers for Disease Control and Prevention (Costerton et al. 1999). Bacterial biofilms are a major problem in various other fields such as fuel storage, water storage, food-processing devices, and medical equipment. The slimy EPS produced by bacteria acts as protective cover that confers resistance against antibiotics and other adverse conditions. The cure of a disease caused by biofilm-forming bacteria requires prolonged treatment, which leads to antibiotic resistance due to high evolutionary pressure. Thus, there is an urgent need for strategies that can control biofilm-forming microorganisms without causing drug resistance; one of these is to stop the biofilm through inhibiting the communication between microorganisms. The *N*-acyl homoserine lactone (AHL) mediated quorum sensing (QS) system is the main regulator of the expression of virulence factors and biofilm development in pathogenic Gram-negative bacteria. Biofilm development also involves other important factors such as flagellar motility and production of EPS (Kohler et al. 2000; Nalca et al. 2006; Vu et al. 2009). Owing to this, QS inhibitors represent very important drug targets for the design of novel therapeutics (Bjarnsholt & Givskov 2007).

Quorum quenching (QQ) is the term used for all processes that interfere in QS (Dong et al. 2001). There are two modes of QQ; firstly, by using small molecules such as QS inhibitors and secondly, the enzymatic degradation of AHL (Uroz et al. 2009). Three enzymes have been reported to cause this degradation; these are AHL acylases, AHL lactonases, and AHL oxidoreductase (Uroz et al. 2009). The rhizosphere supports a diverse bacterial community, which may possess both QS and QQ abilities. For example, AHL-degrading bacteria from ginger and tobacco roots also have QS ability (D'Angelo-Picard et al. 2005; Chan et al. 2011). The QQ activities of the rhizospheric bacteria from cucumber and potato have also been reported (Kang et al. 2004; Jafra et al. 2006). It should be noted that the QQ abilities of these bacteria have been studied mainly for applications as biocontrol agents. QS inhibitors are anticipated to be promising anti-biofilm agents because of the apparent role of QS in biofilm formation (Estrela et al. 2009). However, little is known about the relationship between the anti-biofilm effects of QS inhibitors (Brackman et al. 2011).

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Pseudomonas aeruginosa is a pathogenic opportunistic human pathogen that causes infection through biofilm in patients suffering from cystic fibrosis, which may lead to mortality (Moreau-Marquis et al. 2008). QS in *P. aeruginosa* involves AHLs as signaling molecules (Pearson et al. 1994). The infectious biofilm of *P. aeruginosa* is controlled by QS, which involves AHL. Therefore, QQ in these circumstances would be helpful in combating the disease by the destruction of the biofilm.

In the present study, the extract of a rhizospheric bacterium, *Stenotrophomonas maltophilia* BJ01, is reported to possess QQ ability. The active substance in the extract was identified as *cis*-9-octadecenoic acid using gas chromatography–mass spectroscopy (GC–MS) and electron-spray ionization–mass spectroscopy (ESI–MS). It also inhibited biofilm formation by *P. aeruginosa.* The destruction of biofilm architecture was further confirmed by scanning electron microscopy (SEM) and atomic force microscopy (AFM). The QS inhibition potential of *S. maltophilia* BJ01 could be explored for controlling the virulence of *P. aeruginosa.*

Materials and methods

Isolation and identification of the rhizospheric bacterium

Bacteria were isolated from the roots of *Cyperus laevigatus* L., collected from the coastal region of Dwarka, India (latitude N 21° 51′37.29″, longitude E 69° 21′ 20.79″) using nutrient broth (NB) and agar. Identification of the isolated rhizospheric bacterium was performed by fatty acid methyl ester profiling using MIDI (GC system – 6850, Agilent technologies, USA) and the 16S rRNA gene sequence, amplified by universal primers (fD1-5′-AGA GTT TGA TCC TGG CTC AG -3′ and rP2-5′-ACG GCT ACC TTG TTA CGA CTT -3′) (Weisburg et al. 1991). For whole cell fatty acid analysis, the isolate was grown in Tryptic soy yeast agar for 24 h at 30 °C. Thereafter, preparation and identification of fatty acid methyl esters was done as per the instructions of the Microbial Identification System (MIDI; Microbial ID). Peaks were identified using the RTSBA6 6.10 database (Gontia et al. 2011).

Bacterial strains and growth conditions

P. aeruginosa ATCC 9027 was obtained from HiMedia Laboratories Pvt. Ltd (Mumbai, India). *Chromobacterium violaceum* CV026 was kindly provided by Prof. Anton Hartmann, Helmholtz Zentrum (München, Germany). *S. maltophilia* BJ01 and *C. violaceum* CV026 were grown in NB (Himedia, India) at 30 °C and 180 rpm, while *P. aeruginosa* ATCC 9027 was grown in NB (Himedia, India) at 37 °C and 180 rpm.

Bioassay for QQ by bacterial culture

QQ activity screening was performed using *C. violaceum* CV026 as the reporter strain. The production of a violet pigment in the presence of AHL by strain CV026 is a QS-regulated phenomenon. Five hundred microliters of an overnight culture of *C. violaceum* CV026 were inoculated in 20 ml of NB medium and incubated at 30 °C, shaking at 180 rpm for 2–3 h until the optical density was 0.7 at 600 nm. Ten milliliters of the culture ($OD_{600\ nm} = 0.7$) were added to 150 ml of NB soft agar (maintained at 45 °C) that had been supplemented with *N*-hexanoyl homoserine lactone (HHL) (Sigma–Aldrich, USA). The final concentration of AHL was $0.0625\ \mu g\ l^{-1}$. The bacterial culture was spotted onto an agar plate. After overnight incubation of the plates at 30 °C, the spot surroundings were examined for inhibition of the reporter violacein. Cinnamaldehyde (Sigma–Aldrich, USA) was used as the positive control, while methanol was used as the negative control (Milton et al. 1997). The antibacterial activity of *S. maltophilia* BJ01 was assayed using Mueller–Hinton agar (MHA) following Choo et al. (2006). An antibiotic (tobramycin) was used as the positive control to compare growth inhibition. The surroundings of the paper disc were examined for the presence of a zone of clearance. All experiments were performed in triplicate.

Preparation of extract of S. maltophilia *BJ01*

The QQ compounds were extracted from strain BJ01 using different polar and non-polar solvents (MilliQ water, phosphate buffered saline (PBS), ethyl acetate, hexane, and methanol). These extracts were further evaluated using the above-mentioned plate-based bioassay. To observe whether the production of the QQ compound was induced by AHL or whether its formation was constitutive, an experiment was set up with the addition of AHL in the medium at the time of inoculation.

The extracts were prepared by inoculating an overnight grown culture of strain BJ01 in 100 ml of NB with varying concentrations of AHL ($0.03–0.12\ \mu g\ ml^{-1}$) and incubating for 16 h (30 °C and 180 rpm). Then, the bacterial culture was centrifuged for 10 min at $9500 \times g$ and 4 °C and the supernatant was discarded. The pellet was dissolved in 5 ml of different solvents (MilliQ water, PBS, ethyl acetate, hexane, and methanol), sonicated with a probe for 5 min (Sonics & Materials, Inc VC505, USA) and centrifuged for the removal of cell debris. Next, the supernatant was filtered through a 0.2 μm filter to ensure complete removal of bacterial cells. These extracts were used in the plate-based bioassay of QQ compounds using *C. violaceum* CV026 and to assess the anti-biofilm activity against *P. aeruginosa* ATCC 9027.

QQ bioassay by bacterial cell extracts

The cell extracts of strain BJ01 in different solvents (MilliQ water, PBS, ethyl acetate, hexane, and methanol) obtained from cultures grown with and without the AHL supplement were screened for their QS inhibition ability by using the above-mentioned bioassay method with *C. violaceum* CV026 as the reporter strain.

Purification and characterization of the active compound (GC–MS and ESI–MS)

The crude methanol extract was purified using a solid phase extraction cartridge (Agilent, USA) conditioned with 1 ml of methanol, 5 ml of purified water, and 1 ml of purified, acidified water (pH 2.0) before loading with 200 µl of the sample. The cartridges were than eluted 10 times consecutively with 200 µl of the eluent with different methanol concentrations (from 10 to 100% v/v methanol in water). Each fraction was tested for QQ activity using the bioassay. The active fractions were pooled, evaporated, and dissolved in methanol. GC–MS analysis of the purified extract was carried out on a Shimadzu GC-2010 gas chromatograph fitted with an RTX 1 column. A split-splitless injector heated to 250 °C and a flame ionization detector at 220 °C were used. The oven temperature was programmed as follows: initial temperature of 100 °C for 5 min, increased to 250 °C at the rate of 3 °C min^{-1}, 5 min in 250 °C, increased to 280 °C at the rate of 0.2 °C min^{-1}, and finally 10 min in 280 °C. Helium was used as the carrier gas at a flow rate of 0.94 ml min^{-1}. The injection volume was 1.0 µl (split ratio 1:30). The GC–MS operating parameters were as follows: ionization potential −70 eV with an ion source temperature of 220 °C and scan speed of 1111 amu s^{-1} (scan range 60–600 amu). The identification of the active compound was based on the mass spectra of unknown and reference compounds in a mass spectra library. To further confirm the identity of the compound, commercially available standard extra-pure *cis*-9-octadecenoic acid (Loba Chemie, India) was also subjected to GC–MS analysis using the same parameters. The mass spectra of the purified extract and standard *cis*-9-octadecenoic acid were obtained by ESI–MS in positive mode and acquisition mode scanning from *m/z* 0 to *m/z* 500 (Q-Tof micro ™, Micromass, UK).

Extraction and quantification of violacein

A flask incubation assay was performed to quantify the activity of the QQ compound from the bacterial strain BJ01 extract at different concentrations. *C. violaceum* CV026 was incubated for 16 h in NB Hiveg (Himedia, India) and diluted to 0.1 OD and supplemented with hexonyl-homoserine lactone (0.0625 µg ml^{-1}) and extracts at different concentrations ranging from 0.462 to 3.696 mg ml^{-1} or (0.462, 0.924, 1.848, and 3.696 mg ml^{-1}). The experiment was also performed with pure *cis*-9-octadecenoic acid (3.696 mg ml^{-1}). The flasks were incubated at 30 °C and shaking at 180 rpm for 24 h. One milliliter of the culture from each flask was centrifuged (16,000 × *g* for 10 min) for the precipitation of insoluble violacein. The culture supernatant was discarded and 1 ml of dimethylsulfoxide (DMSO) was added to the pellet. The solution was vortexed vigorously for 30 s to completely solubilize the violacein and centrifuged at 16,000 × *g* for 10 min to remove the cells. Two hundred microliters of the violacein-containing DMSO were added to 96-well flat bottomed microplates (Axygen, India), 3 wells for each solution, and the absorbance was read with a microplate reader (Spectra Max plus, USA) at a wavelength of 585 nm (Choo et al. 2006; Zhu et al. 2011).

Heat treatment

To determine the thermal stability of the QQ compound, the extract was kept at different temperatures (40, 50, and 60 °C) for 30 min in a water bath and at 121 °C (autoclaved). Autoclaved pure *cis*-9-octadecenoic acid was used as a positive control. The extract with different temperature treatments and pure *cis*-9-octadecenoic acid (121 °C) were cooled to room temperature and the QS inhibition activity was determined by the quantification of violacein. Violacein production without extract treatment was taken as the control and the inhibition activity by the non-heat treated extract was also determined (25 °C). The experiment was performed with 5 replicates.

Biofilm formation assay

An overnight culture of *P. aeruginosa* ATCC 9027 in NB, diluted to 0.1 OD at 620 nm, was added to a 96-well polystyrene microtiter plate along with different concentrations of the strain BJ01 extract (0.147, 0.295, 0.443, 0.591, 0.739, and 1.478 mg ml^{-1}) and pure *cis*-9-octadecenoic acid (200 µl $well^{-1}$ and 5 wells $sample^{-1}$). The plate was incubated for 24 h (100 rpm, 30 °C). The biofilm grown without the extract was served as the control. Growth was measured at 620 nm. The wells were washed, dried, and stained with 1% crystal violet. Excess dye was washed off. Thereafter, 200 µl of 96% ethanol were added, and the absorbance was measured at 590 nm. The experiment was performed with 5 replicates (Andersson et al. 2009).

To evaluate the effect of the extract on cell viability, cells within the biofilm were labeled with a fluorescent dye using the FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Invitrogen, USA) and visualized under an epifluorescence microscope (Axio Imager, Carl Zeiss AG, Germany). The samples were prepared according to the manufacturer's instructions. Live cells were stained green

by SYTO-9 and dead cells were stained red by propidium iodide.

Visualization of biofilm inhibition by SEM

SEM was used to study the *P. aeruginosa* ATCC 9027 biofilm in the presence or absence of the extract using a modification of the method of Andersson et al. (2009). Briefly, a biofilm of *P. aeruginosa* ATCC 9027 was grown on glass coverslips submerged in NB with different concentrations (0.147, 0.295, 0.443, 0.591, 0.739, and 1.478 mg ml^{-1}) of the extract. Samples without the extract and with pure *cis*-9-octadecenoic acid (1.478 mg ml^{-1}) served as the negative and positive controls, respectively. After incubation for 24 h, the medium was removed and the coverslips were gently washed with 0.9% NaCl to remove planktonic cells. The samples were prefixed in 2.5% glutaraldehehyde in H_2O (20 min), and post-fixed for 30 min in 4% OsO_4 in 0.1 M phosphate buffer. The biofilms were dehydrated in a graded ethanol and hexamethyl disilazan series (10–95%) for 10 min at each concentration. The dried biofilms were then coated with gold before observation under a SEM (LEO series VP1430, Germany) with an accelerated voltage of 20 kV.

Atomic force microscopy

The sample for AFM was prepared by submerging sterile glass coverslips (11 mm in diameter) in a 24-well polystyrene plate with NB containing a bacterial suspension of 1% inoculum with an OD of 0.1 (diluted from an overnight grown culture). The bacterial extract was added at a concentration ranging from 0.147 to 1.478 mg ml^{-1}in each well and pure *cis*-9-octadecenoic acid was used as the positive control. The plate was incubated for 24 h at 30 °C and 100 rpm. The biofilm on the glass surface was rinsed gently with PBS (pH 7.4) and kept under vacuum until it was completely dry. The biofilm was observed under AFM without staining to substantiate the significant reduction in biofilm. Scanning was performed at a scan speed of 1 Hz and in semicontact mode. The WS × M image viewer was used to analyze the topographic images of the surface (NT-MDT, Russia) (Oh et al. 2009; Nithya et al. 2010).

Motility and pyocyanin assay

The swarming motility test was performed using an overnight culture (OD_{600nm} = 1.0) of *P. aeruginosa*. The cells were grown in BM2 swarming medium (62 mM PBS at pH 7, 2 mM $MgSO_4$,10 µM $FeSO_4$, 0.4% glucose, 0.1% casamino acids, and 0.5% agar) supplemented with or without the extract and pure *cis*-9-octadecenoic acid (1% v/v) (Overhage et al. 2007).

The extract or the pure compound was added to 5 ml of PB medium (20 g peptone, 1.4 g of $MgCl_2$, and 10 g of K_2SO_4 l^{-1} of distilled water) containing 1% *P. aeruginosa* culture and incubated for 18 h at 37 °C. The culture supernatants were extracted with 3 ml of chloroform and then re-extracted into 1 ml of 0.2 N HCl to obtain a pink to deep red solution. The absorbance of the solution was measured spectrophotometrically at OD_{520} to determine the pyocyanin content (Essar et al. 1990).

Statistical analysis

Statistical analysis was performed using Sigmaplot 12. All values are expressed as mean ± SD. One-way ANOVA followed by Dunnett's *post hoc* test was applied for the comparison of test samples and controls with a P value of 0.05 considered as significant.

Results and discussion

Isolation and identification of the bacterium

The bacterium was isolated from the roots of *C. laevigatus* L., and the whole cell fatty acid methyl ester profiling showed that the isolated strain belonged to *S. maltophilia*. Fatty acid methyl ester profiling of *S. maltophilia* revealed the presence of 15:0 iso, 15:0 anteiso, and 16:1w7c as the major characteristic fatty acids (Supplementary material Figure S1). [Supplementary material is available *via* a multimedia link on the online article webpage.] The bacterium was further identified by 16S rRNA gene sequence analysis. Using conserved primers (Weisburg et al. 1991), the 16S rRNA gene was amplified, sequenced, annotated, and submitted to NCBI [GenBank: JQ609679] under the name *S. maltophilia* strain BJ01.

There are 8 species of genus *Stenotrophomonas*, of which *S. maltophilia* is the predominant one (Ryan et al. 2009). *S. maltophilia* has been isolated from different plants such as wheat, oat, cucumber, maize, cabbage, and tobacco (Berg et al. 1996; Jin et al. 2011). It is also an opportunistic human pathogenic bacterium (Berg et al. 2005). Various beneficial applications of this Gram-negative bacterium, including biological control of fungal plant diseases, bioremediation, and the production of detergent proteases are known (Ryan et al. 2009; Jin et al. 2011; Ribitsch et al. 2012). The present study reports for the first time the QQ and anti-biofilm activity of *S. maltophilia* isolated from a salt-tolerant grass species.

Bioassay for QQ by S. maltophilia

The QQ property of *S. maltophilia* was studied using *C. violaceum* CV026 as a biosensor. Violacein production is a QS regulated behavior in strain CV026. *S. maltophilia* strain BJ01 showed a white-colored, opaque zone of inhibition (Figure 1a) with intact bacteria, which represents the QQ. The absence of any clear/transparent zone on the sensor plate demonstrated that *S. maltophilia* did not inhibit growth of the CV026 biosensor. To check whether *S. maltophilia* has any antimicrobial activity

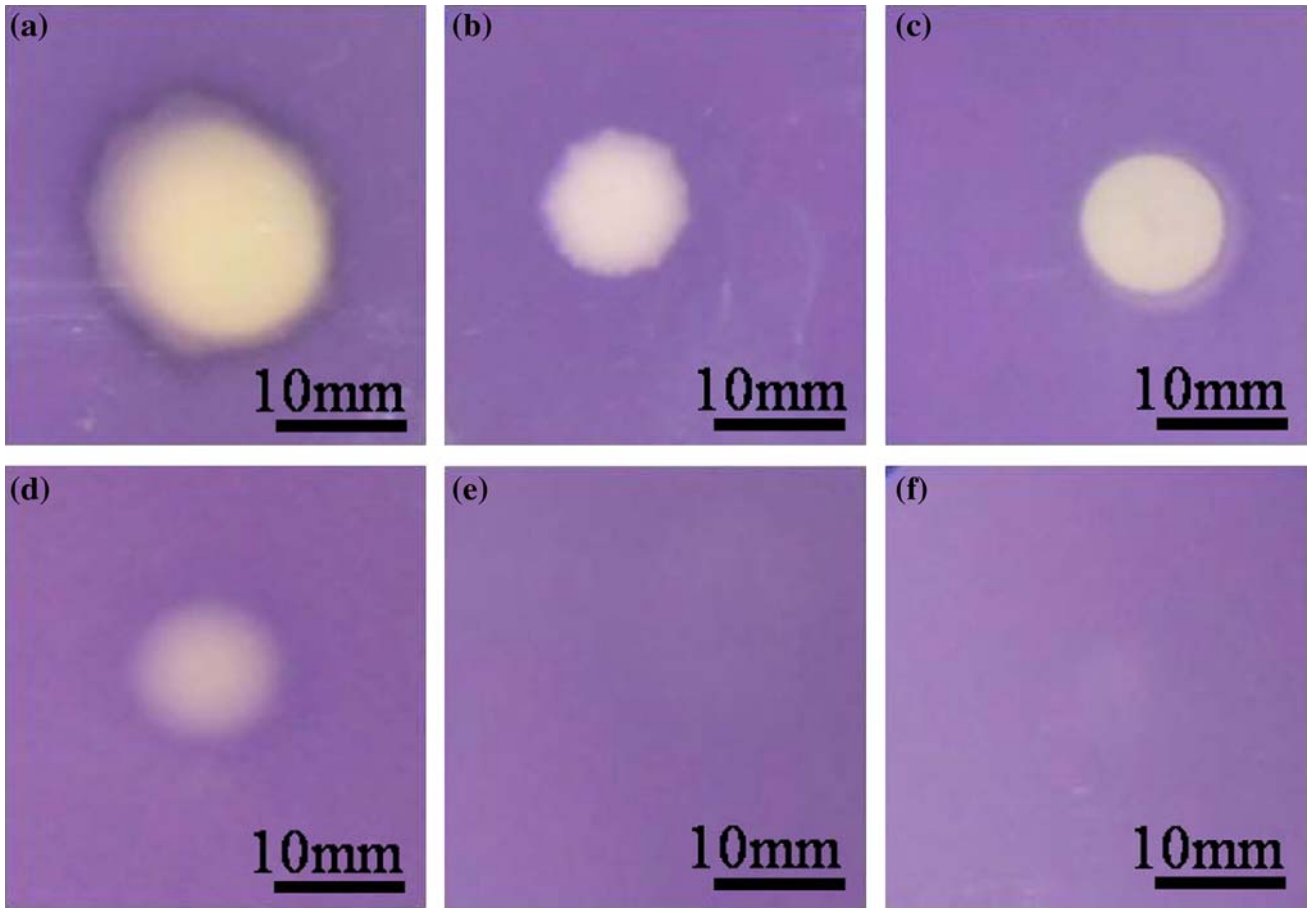

Figure 1. QQ activity of the bacterium and its extract. Inhibition of violacein production by bactrial culture (a), cinnamaldehyde (b) served as a positive control, extract (c) and standard *cis*-9-octadecenoic acid (d) against *C. violaceum* CV026, methanol (e), and phosphate buffer saline (f) served as negative controls.

against CV026, the disc diffusion assay was performed (Supplementary material Figure S2). No clear zone was observed on the MHA plate, indicating that *S. maltophilia* BJ01 did not inhibit the growth of strain CV026. Cinnamaldehyde is a known QS inhibitor whose mechanism of action is speculated to be dependent on its three-carbon aliphatic side chain; this may interfere with the binding of the smaller 3-hydroxy-C4-and 3-oxo-C6-HSLs to their cognate receptors (Niu et al. 2006; Brackman et al. 2008). Therefore, cinnamaldehyde was used as the positive control to compare the extent of inhibition of QS. Cinnamaldehyde showed maximum inhibition at 75 mM (data not shown). The zone of inhibition with strain BJ01 (Figure 1a) was of a larger diameter (15 mm) than that of cinnamaldehyde (Figure 1b) (11 mm).

The QQ property has been reported recently for several rhizospheric bacteria (Christiaen et al. 2011). One of the isolates, MP1-3, showed close identity with *Stenotrophomona rhizosphila* and did show a marginal reduction in AHL levels; however, another isolate, MP1-4, failed to do so (Christiaen et al. 2011). The present result shows that QQ is induced by AHL. It is likely that a lower concentration of AHL induces/enhances the production of *cis*-9-octadecenoic acid, which may interfere with QS. To the best of the authors' knowledge, this is the first report of the QQ ability of a rhizospheric strain of *S. maltophilia*. This was further confirmed by measuring the amount of violacein production, which indicates the inhibition of QS (Choo et al. 2006).

Evaluation of QQ activity of the extract using different solvents

Extracts of *S. maltophilia* were prepared separately in MilliQ water, PBS, ethyl acetate, hexane, and methanol, grown in NB with or without AHL priming (the optimum concentration of AHL was used, ie 0.06 $\mu g\,ml^{-1}$). (Supplementary material Figure S3). Each extract was screened by the plate bioassay in triplicate for QQ activity against *C. violaceum* CV026. The results showed the absence of a zone of inhibition (QQ) when extracts were prepared from the culture grown without AHL. In contrast, QQ was observed with PBS and methanol extracts from the cultures grown with AHL. Figure 1c represents the QQ in terms of the absence of a violet color where

the extract was spotted. Similar white-colored opaque zones were seen with both the PBS and methanol extracts. To confirm that these zones were not due to methanol or PBS, both were spotted separately as negative controls (Figure 1e and f). The complete absence of QQ activity with all extracts (ethyl acetate, hexane, methanol, MQ, and PBS) of *S. maltophilia* grown without AHL strongly suggests that pre-AHL treatment may induce/enhance the production of the quorum quencher. On the other hand, when the cultures were primed with AHL, methanol, and PBS extracts gave positive results (QQ). Ethyl acetate was used to extract the QS inhibitors from some marine bacterial isolates that showed concentration-dependent inhibitory activity (Nithya et al. 2010). However, no QQ activity of *S. maltophilia* was seen when the extract was prepared in ethyl acetate. This result indicates that the QQ compound of *S. maltophilia* may be highly hydrophilic in nature, as it might not be sufficiently soluble in ethyl acetate (Teasdale et al. 2011). To check this further, another non-polar organic solvent, hexane, was used, which showed similar results as ethyl acetate. However, when methanol (a polar organic solvent) was used for the extraction, the extract gave a positive result for QQ. Similarly, other polar inorganic solvents, MilliQ water and PBS, were also tested. Some QQ activity was observed in the PBS (pH 7.4) extract, but not in the aqueous extract.

Characterization of the extract

GC–MS analysis of the purified extract predominantly showed a single peak with a retention time of 13.9 min having a relative abundance of 85.52% (Figure 2a). The MS analysis showed that the peak corresponded to *cis*-9-octadecenoic acid with a molecular formula of $C_{18}H_{34}O_2$

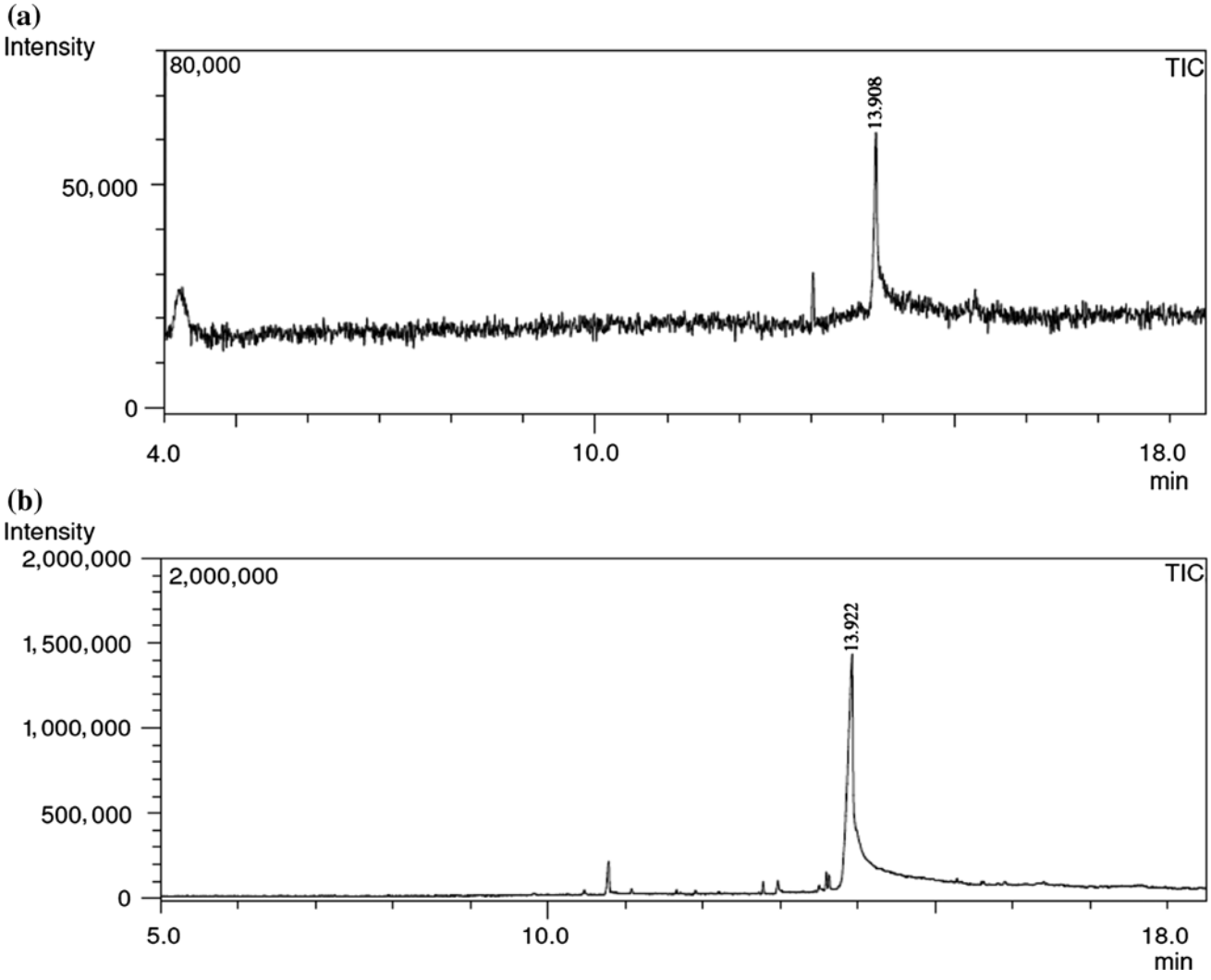

Figure 2. GC chromatograms of a purified methanol extract *S. maltophilia* strain BJ01 (a) and standard *cis*-9-octadecenoic acid (b). By means of mass spectra analysis, the compound was identified as *cis*-9-octadecenoic acid which showed the same chromatogram as that of standard *cis*-9-octadecenoic acid.

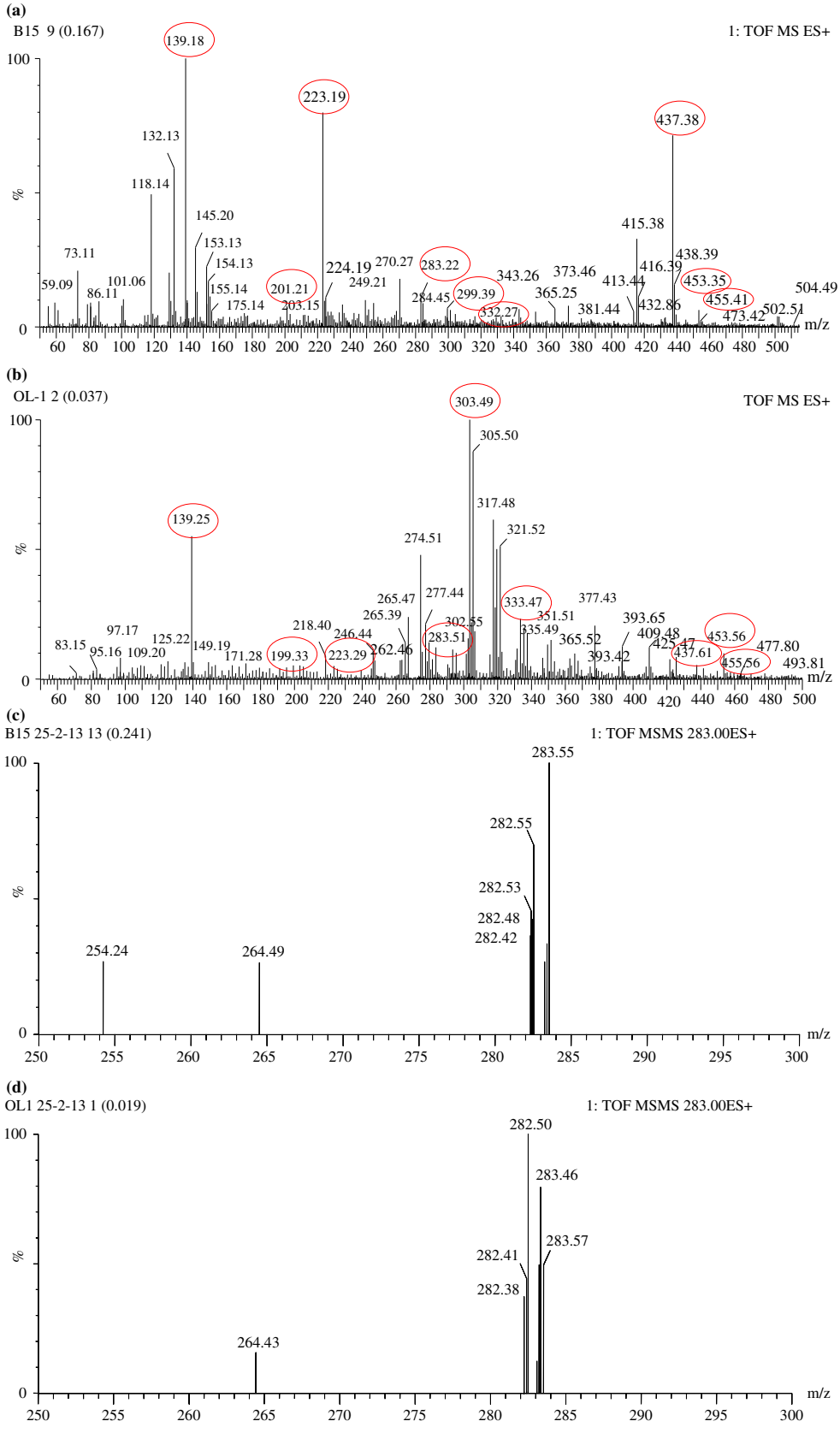

Figure 3. ESI–MS of the purified extract (a) and standard *cis*-9-octadecenoic acid (b). Red circles indicate the common peaks in both the spectra. Additional peaks represent the stable fragmented moieties of the parent molecule. The mass spectra were acquired in positive mode and scanning range from *m/z* 0 to *m/z* 500. The MS–MS spectra of base peak of the extract (c) and standard (d).

by matching with a GC–MS library (NIST 27. LB). To confirm the identity of the compound, it was compared with standard *cis*-9-octadecenoic acid, which showed a peak at the same retention time (13.9 min) (Figure 2b). The ESI–MS data revealed the spectral base peak at *m/z* 283.22 and 283.51 $[M+H]^+$ (where M is *cis*-9-octadecenoic acid) of the sample and standard, respectively, which matched with the calculated value of 283.26 (Figure 3). Additional peaks were also observed in the ESI–MS spectra, which represent the stable fragmented moieties of the parent molecule. The MS–MS spectrum of the base peak of the extract completely matches with that of standard *cis*-9-octadecenoic acid (Figure 3). Thus, the ESI–MS data are in conformity with the results of GC–MS analysis.

Violacein quantification

The QQ property of the methanol extract, observed in the *C. violaceum* CV026-based bioassay was further validated by quantifying violacein production. Violacein production was significantly ($p < 0.05$) inhibited by the methanol extract; the reduction was proportionate to the amount (w/v) of extract. A concomitant reduction in violacein with increasing concentration of the extract was observed. The reduction was 10, 65, 85, and 95% in the presence of 0.462, 0.924, 1.848, and 3.696 mg ml^{-1} of the extract, respectively, and representative results are shown (Figure 4). The percentage reduction obtained with the highest concentration of the extract was comparable with that of the pure compound (94%) (Figure 4). Fatty acids, including *cis*-9-octadecenoic acid (oleic acid), are known to inhibit the QS signal autoinducer-2 (AI-2) (Widmer et al. 2007). In this report, it has been shown that *cis*-9-octadecenoic acid significantly inhibited ($p < 0.05$) AHL-mediated QS. Halogenated furanones have been reported to inhibit AI-2 (Ren et al. 2004) as well as AHL-mediated QS (Manefield et al. 2002). It is presumed that the acid functionality of *cis*-9-octadecenoic acid may interact with LuxR, hampering its function. Some plant and fungal natural products showing QS inhibition against C. *violaceum* CV026 have been reported (Delalande et al. 2005; Choo et al. 2006; Zhu & Sun 2008; Lee et al. 2011; Zhu et al. 2011). It is difficult to exactly compare the results because the methods of extraction differ significantly. However, it is worth mentioning that a vanilla bean extract reduced violacein production in a concentration-dependent manner (Choo et al. 2006). Recently, it has been shown that a methanol extract of an edible plant, *Melicope lunu-ankenda*, inhibited the response of *C. violaceum* CV026 to HHL (Tan et al. 2012). However, significant inhibition was observed only when the extract was used at a very high (4 mg ml^{-1}) concentration.

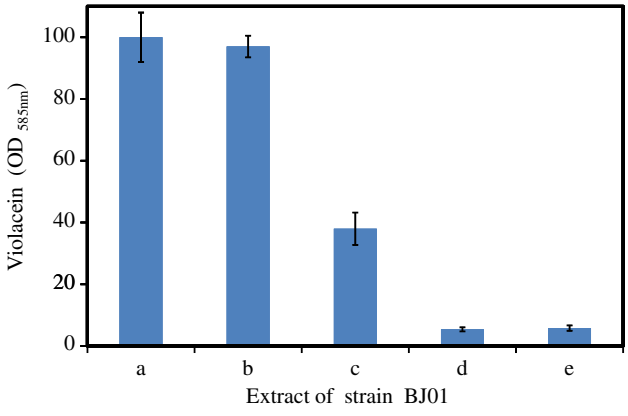

Figure 4. Extract of strain BJ01 inhibits production of violacein by *C. violaceum* CV026 in a concentration dependent manner. Inhibition of violacein production was measured without extract; treated as control (a), with methanol as negative control (b), with 0.924 mg ml^{-1} (c), 3.696 mg ml^{-1} (d) of methanol extract of strain BJ01, and 3.69 mg ml^{-1} of standard *cis*-9-octadecenoic acid (e). The data represented are average ± SD of the absorbance at 585 nm. Analysis of data by Dunnett's test demonstrates significant difference between the tests and the control ($p < 0.05$).

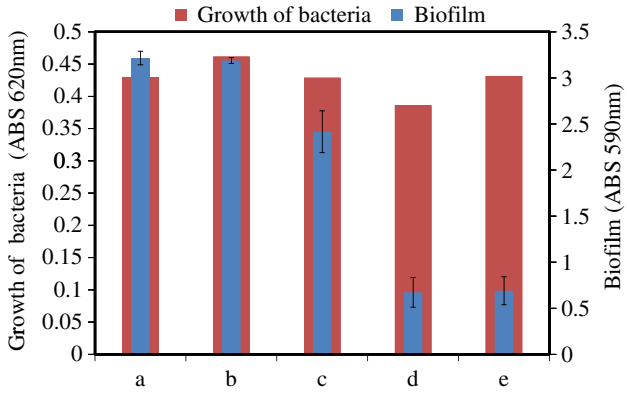

Figure 5. Extract of *S. maltophilia* BJ01 inhibits *P. aeruginosa* biofilm formation in a concentration dependent manner. Biofilms were developed along with different concentrations of extract in 96 wells microtiter plate. The growth was represented by red bars and biofilm was represented by blue bars. Biofilm formation was measured without extract (a), with methanol as negative control (b), with 0.591 mg ml^{-1} of extract (c), 1.478 mg ml^{-1} of extract (d), and pure *cis*-9-octadecenoic acid (e). Data shown are averages ± SD. The error bars represent ± SD of mean value (five replicates). Analysis of data by one way ANOVA followed by Dunnett's test demonstrates significant difference ($p < 0.05$) between biofilm of the tests and the control. No significant difference was observed in cell growth between treated and untreated control.

QQ activity of the extract after heat treatment

The QQ ability of the heat-treated extract of *S. maltophilia* was studied; the extract concentration used was 3.696 mg ml^{-1}, as this provided the maximum inhi-

bition. An increase in the temperature caused a significant ($p < 0.05$) restoration of violacein production. Degradation was observed at 40 °C (12.38%), which increased and became constant at 50 °C (23.91%) and 60 °C (24.20%). Approximately, 60% of violacein was restored compared to the control when treated with the autoclaved extract (121 °C) (Supplementary material Figure S4). The results were compared with the control (without the extract) and the extract at 25 °C (RT) (Supplementary material Figure S4). The results reveal that an increase in temperature causes a reduction in QQ compound activity. However, even at the highest temperature, it was not fully degraded. The residual QQ activity suggests partial heat stability. Such partial heat inactivation has recently been observed in other rhizospheric bacteria belonging to the genera *Anthrobacter*, *Pseudomonas,* and *Delftia* (Christiaen et al. 2011).

Evaluation of anti-biofilm activity

The extract of strain BJ01 showed significant ($p < 0.05$) inhibition of biofilm formation without affecting the growth of *P. aeruginosa* ATCC 9027 (Figure 5). Spectrophotometric data of growth analysis of *P. aeruginosa* with different concentration of the extract substantiated that the growth was not significantly affected ($p < 0.05$) by the addition of the extract. However, the biofilm of *P. aeruginosa* was significantly reduced with an increasing concentration of the extract. The representative results are shown in Figure 5. These results clearly indicate that the observed biofilm inhibition by the extract of strain BJ01 was not due to toxicity. These results are in agreement with the previous finding of *P. aeruginosa* biofilm inhibition by some of the marine *Bacillus* spp. (Nithya et al. 2010). This is the first report of the anti-biofilm activity

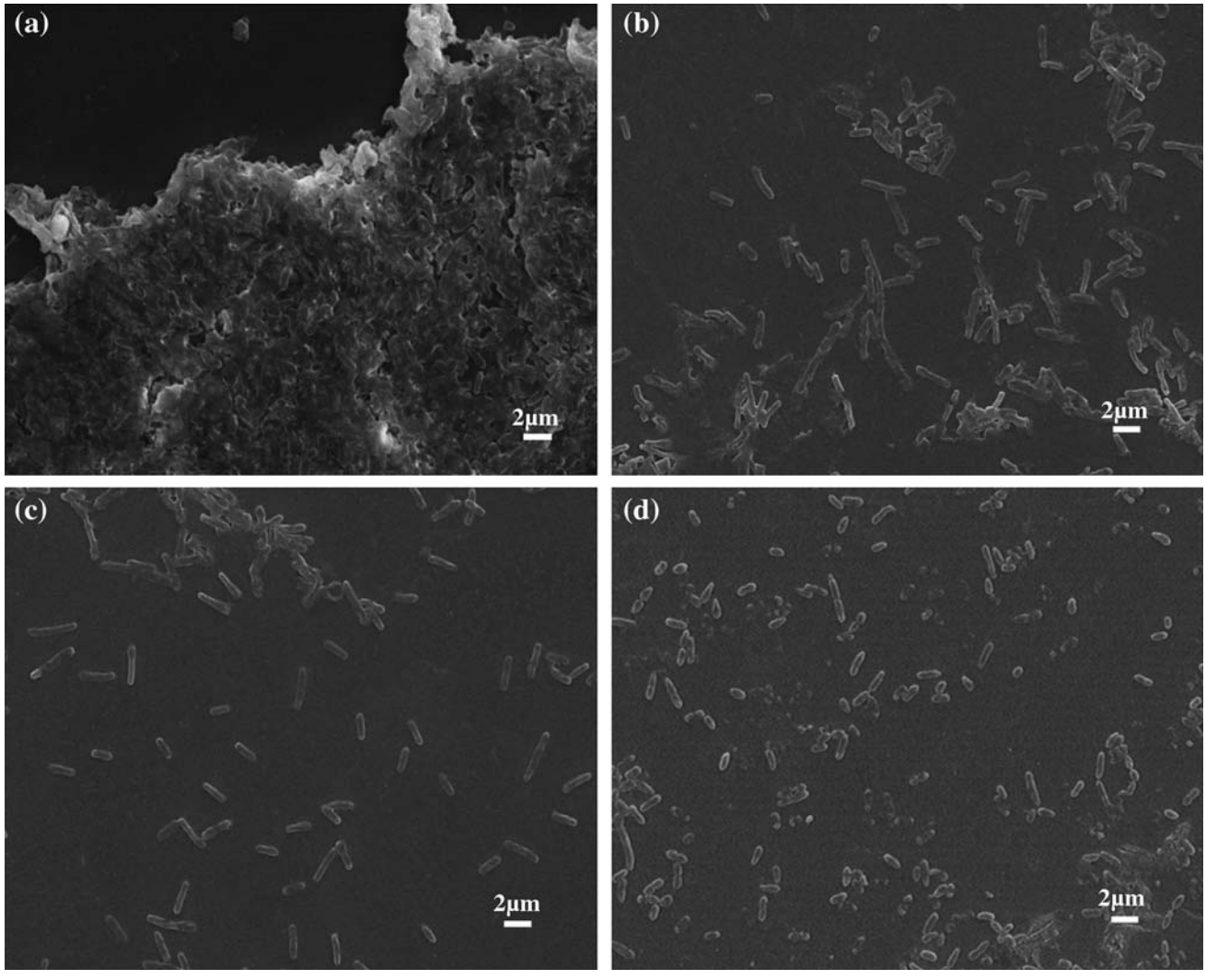

Figure 6. SEM images illustrating the effects of different concentrations of extract on *P. aeruginosa* ATCC 9027 biofilm. Biofilms were formed on coverslips within 24 h at 30 °C. The biofilms were treated with extract at 0.147, 0.295, 0.443, 0.591, 0.739, or 1.478 mg ml^{-1} at 30 °C and representative figures are shown as (a) control (untreated), (b) treated with 0.591 mg ml^{-1}, (c) 1.478 mg ml^{-1} of extract, and (d) standard *cis*-9-octadecenoic acid.

of *cis*-9-octadecenoic acid. To check the effect of the extract on cell viability, the biofilm was stained with live/dead dyes. The extract did not reduce (Supplementary material Figure S5) the viability of the cells.

Cis-9-octadecenoic acid is a derivative of *cis*-2-decenoic acid. The anti-biofilm activity of *cis*-9-octadecenoic acid may be attributed to its structural and functional resemblance to short-chain fatty acid signaling molecules, such as diffusible signal factor (cell-to-cell communication molecules in bacteria) (Dow et al. 2003). *Cis*-2-decenoic acid has been reported to induce dispersion in microbial biofilms of bacteria such as *P. aeruginosa* PA01, *Escherichia coli*, *Klebsiella pneumonia*, *Proteus mirabilis*, *Streptococcus pyogenes*, *Bacillus subtilis*,

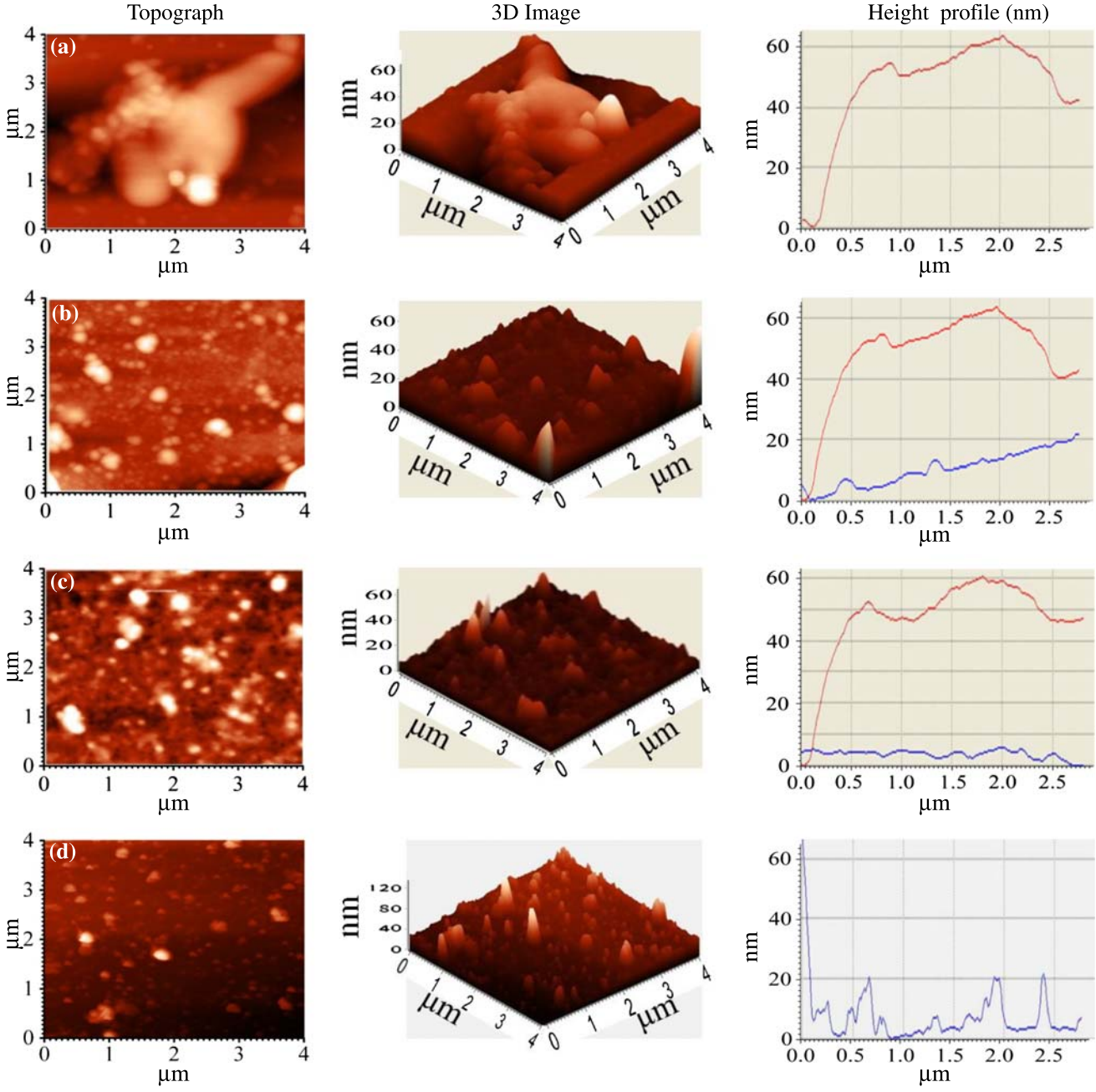

Figure 7. AFM images illustrating the effects of different concentration of extract on *P. aeruginosa* ATCC 9027 biofilm (topography, 3D image, and height profile). Biofilms were grown on coverslips for 24 h at 30 °C. The biofilms were treated with extract at 0.147, 0.295, 0.443, 0.591, 0.739, or 1.478 mg ml^{-1} at 30 °C and representative figures are shown as (a) control (untreated), (b) treated with 0.591 mg ml^{-1}, (c) 1.478 mg ml^{-1} of extract, and (d) standard *cis*-9-octadecenoic acid. Scanning was performed at a scan speed of 1 Hz and at semicontact mode. The WS × M image viewer was used to analyze topographic images of the surface. *X*-axis represents distance and *Y*-axis represents height (nm).

Staphylococcus aureus, and the yeast *Candida albicans* (Davies & Marques 2009). A branched-chain fatty acid, 12-methyltetradecanoic acid (anteiso-C15:0), has also been reported to repress biofilm formation in *P. aeruginosa* PA01 (Inoue et al. 2008). Moreover, sugar fatty acid esters with long-chain fatty acid residues (C14–16) have been reported to inhibit biofilm formation by *Streptococcus mutans*, *Listeria monocytogenes,* and *P. aeruginosa* (Furukawa et al. 2010). Thibane et al. (2010) reported that marine polyunsaturated fatty acids are involved in the inhibition of *Candida* biofilms.

The biofilm mode of life of bacteria is associated with increased resistance towards antibacterial agents, making successful treatment of biofilm-related infections difficult (Høiby et al. 2010). Bacterial social life is dependent on 2 interrelated phenomena: QS and biofilm formation (Nadell et al. 2008). Owing to this, it is not surprising that quorum quenchers could also inhibit biofilm formation. Recently, it has been shown that a commercially available QQ compound could increase the susceptibility of bacterial biofilm to antibiotics both *in vitro* and *in vivo* (Brackman et al. 2011). This may be used for the effective treatment of biofilm-related infections as QQ facilitates the activity of antibiotics on biofilm-forming bacteria.

SEM evaluation of biofilm inhibition

The biofilm inhibitory activity of the extract of *S. maltophilia* BJ01 at different concentrations (0, 0.147, 0.295, 0.443, 0.591, 0.739, and 1.478 mg ml^{-1}) was observed against *P. aeruginosa* ATCC 9027 using SEM. Fully grown biofilms with bacterial cells embedded in EPS on a glass surface were observed in the absence of the extract (control) (Figure 6). Biofilm formation decreased with increasing concentrations of the extract as compared to the control and representative results are shown in Figure 6. The steady decrease in the biofilm was associated with a decrease in EPS. The bacteria were in the discrete form rather than in a group. Almost complete inhibition of the biofilm was observed when 1.478 mg ml^{-1} extract was applied (Figure 6c). This is in conformity with the results obtained in the biofilm formation assay (Figure 5). The biofilm acquires a characteristic architecture during the course of its development (You et al. 2007). The extract from strain BJ01 disrupted the architecture of the biofilm and separated the cells. The destruction of biofilm architecture provides an attractive anti-biofilm strategy against drug-resistant *P. aeruginosa.*

Atomic force microscopy

The disruption of the *P. aeruginosa* ATCC 9027 biofilm by the extract was further checked using AFM. Biofilm disruption was evaluated in terms of the difference in the surface roughness and the average height. The root mean square average height is the mean value of the surface relative to the difference in height between the maximum and the minimum data points on the surface with respect to the mean plane. The topography of the biofilm treated with different concentrations of the extract showed decreased adherence of cells onto the surface compared to the control (Figure 7). The thickness of the height profile revealed a significant decrease in adherence to the surface. The extract at 0.147, 0.295, 0.443, 0.591, 0.739, and 1.478 mg ml^{-1} resulted in an average biofilm height of 50, 40, 20, 20, 15, and 5 nm, respectively, against the control at 60 nm. Representative images are shown in Figure 7. Extract treatment also affected the topography of the *P. aeruginosa* biofilm. This observation confirmed the results of the SEM analysis.

Effect on swarming motility and pyocyanin production

Apart from biofilm inhibition, it is equally important to know whether the extract could inhibit other QS regulated phenomena such as swarming motility and production of the virulence factor pyocyanin. Motility behavior was assessed by the swarming motility assay. Treated (with the extract or the pure compound) cells of *P. aeruginosa* showed less flagellum-driven motility on agar plates compared to the untreated control (Supplementary material Figure S6). The pyocyanin assay revealed that the extract and pure *cis*-9-octadecenoic acid inhibited pyocyanin production by 46.33 and 50.97% ($p < 0.005$), respectively (Supplementary material Figure S7). The production and regulation of swarming and pyocyanin are controlled by the *rhl* system in *P. aeruginosa* (De Kievit & Iglewski 2000; Kohler et al. 2000). Thus, the observed inhibition of *P. aeruginosa* swarming motility and pyocyanin production suggests that *cis*-9-octadecenoic acid may act as an *rhl* inhibitor.

Conclusions

The bacterium isolated from the rhizosphere of *C. laevigatus* L. was identified as *S. maltophilia* BJ01 using the whole cell fatty acid profile and 16S rRNA gene sequence homology. The methanol extract of *S. maltophilia* BJ01 showed significant QQ activity against *C. violaceum* CV026 and anti-biofilm properties against pathogenic *P. aeruginosa* without affecting its growth. The active substance in the extract was identified as *cis*-9-octadecenoic acid on the basis of GC–MS and ESI–MS. This is the first report of the QQ and anti-biofilm activity of bacteria-derived *cis*-9-octadecenoic acid. *Cis*-9-octadecenoic acid may have potential medical applications in controlling detrimental infections with *P. aeruginosa*.

List of abbreviations

QS	quorum sensing
AHL	acyl homoserine lactone
QQ	quorum quenching
ESI–MS	electrospray ionization–mass spectrometry
GC–MS	gas chromatography–mass spectrometry

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